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13. ABSTRACT ***(Maximum 200 Words)***

The general goal of this study was to determine if there are specific combinations of oligosaccharide markers on ductal breast carcinoma cells that are useful in predicting the post surgical prognosis of low-risk node-negative breast cancer patients. A related goal was to define the expression of relevant glycosyltransferase genes that control the expression of unusual tumor-associated oligosaccharides found on breast cancer cells. Using a panel of monoclonal antibodies specific for the oligosaccharides Le^a, sialyl-Le^a, extended Le^a-Le^x, Tn antigen, T antigen and also Cathepsin D, we found no statistically significant association of the expression of these antigens and the recurrence risk among 181 low-risk, post-surgical breast cancer patients. Studies of cloned cancer-cell lines that overexpressed Le^a-Le^x resulted in the cloning of four genes that can either suppress or enhance Le^a-Le^x expression. Two of these have sequence homology to known fucosyltransferase genes, but have unique sequences. These studies also showed that the expression of several tumor-associated cell-surface oligosaccharides is regulated by cell-cell contacts with neighboring cancer cells. For example Le^a-Le^x cannot be detected on the cell surface unless contacts are made with adjacent cancer cells.

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David E. Pettijohn 9-29-99

PI - Signature Date

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INTRODUCTION

Patients who have small node-negative ductal breast carcinomas generally have a favorable prognosis (see ref. 1 for review). After surgery, relapse occurs in less than 20% of these so-called low-risk patients during the following 10 year period. In spite of this favorable prognosis, the management of these low-risk patients can be complicated, as there is no established way to identify the 20% who will relapse. The patients who could benefit most from adjuvant chemotherapy cannot be reliably predicted and equally important, the patients who don't require post-surgical adjuvant therapy cannot be identified. The purpose of the present research was to devise an approach exploiting unusual oligosaccharide cell-surface markers that appear on breast cancer cells to identify the low-risk patients who have poor prognosis. A further goal is to determine which genes are involved in the aberrant expression of the unusual oligosaccharides found on some but not all cells of ductal breast carcinomas.

The overall approach of these studies is to determine if there are specific combinations of oligosaccharide markers and other makers on breast cancer cells that are useful in predicting the post surgical prognosis of low-risk node-negative breast cancer patients. The markers identified from these studies would then be combined with other known prognostic markers in an attempt to assemble a set of markers which could indicate with highest specificity and sensitivity the patients who are at greatest risk for relapse. The studies are also intended to identify glycosyltransferase activities that may be expressed in certain carcinomas that are correlated with poor prognosis. This identification would open the way for new approaches to studying the biological effects of the most significant oligosaccharides.

BODY

A large group of breast tumor specimen was obtained from a collection of the Danish Breast Cancer Cooperative Group, which is a nationwide surveillance and research program (2). All specimen were from women who had low-risk node negative ductal breast carcinomas and who had surgery 5-20 years previously and who have been closely followed since surgery. None of the women had chemotherapy, so that the prognosis is unaffected by other post-surgical interventions. A panel of well-characterized monoclonal antibodies with known specificity for specific oligosaccharides was employed to define the cell surface oligosaccharides and proteolytic activities (such as Cathepsins) associated with the tumor cells. After completing the survey, the relapse history of the patients was compared with the different molecular markers using Cox's proportional hazards model (3) to identify statistically significant independent markers of prognosis. Potentially, it would then be possible to select different combinations of markers to attempt to improve specificity and sensitivity by using a panel of prognostic markers.

Task 1. We tested the possibility that the Le^a-Le^x oligosaccharide marker, present or absent on the cancer cells from low-risk ductal breast carcinoma patients, was correlated with post-surgical relapse frequency or with the number of years of post-surgical survival. For this study we used imunofluorescent staining of tumor sections with monoclonal antibodies specific for Le^a-Le^x. As background for this research we continued our studies of the natural occurrence of Le^a-Le^x which in earlier animal model research had first indicated that this oligosaccharide could be a useful prognostic marker (4). We found that Le^a-Le^x is frequently expressed in tumors derived from cells of embryonic endoderm lineage, but found no examples where expression occurred in tumors with other lineages (5) (see manuscript of ref. 5 in Appendix for details).

Initially, it was intended to examine tumor specimen from 260 low-risk ductal breast carcinoma patients, but after careful screening of specimen and clinical records, it was determined that only specimen from 181 patients were actually available. The others were eliminated from the study because of one of the following reasons: a) the tissue block was exhausted; b) the patient was later shown to have nodal involvement or other metastatic lesions and thus was disqualified from inclusion; c) more detailed examination of clinical records showed that the patient had received radiation or chemotherapy and therefore could not be included in the study. Initial results from the first 86 specimen indicated a positive correlation between the expression of Le^a-Le^x and relapse (see ref. 6 for abstract of a presentation at a national meeting). However later data from the subsequent specimen did not support this interpretation. Results from detailed statistical analyses and Kaplan-Meier plots are shown in Appendix Table 1 and Figs. 2 plus 3. The only tumor variable that had a statistically significant correlation with relapse free survival was tumor size.

As noted in an earlier progress report, we were puzzled by the apparent inconsistency between these results and the earlier Le^a-Le^x findings. For this reason we re-examined all available specimens and during the last year confirmed the lack of statistically significant correlation.

Task 2. We determined if other oligosaccharides related to Le^a-Le^x could be used as prognostic markers for patients with low-risk ductal breast carcinomas. For this analysis we used immunofluorescence and monoclonal antibodies specific for Le^a, Sialyl-Le^a, Tn antigen, Sialyl-Tn, and Le^x. Each of these markers like Le^a-Le^x is variably present on cells of different ductal breast carcinomas; however the presence or absence of none of them was correlated with relapse frequency or survival (Appendix Table 1 and Figs 2 + 3). Likewise, the Biostatistics Core Laboratory of the University of Colorado Cancer Center used methods for correlating recurrence-free survival simultaneously with several marker variables and was unable to assign a statistically significant correlation with any two simultaneous variables.

During the course of these studies, Dr. Ulla Engel from the State University Laboratory, Copenhagen was a visiting research scientist in our laboratory. She had joined us to study the expression of our panel of cell surface oligosaccharides in esophageal adenocarcinomas. Using the same immunofluorescence methods and our panel of monoclonal antibodies, she demonstrated that those adenocarcinomas arising in Barrett's epithelium could be distinguished by a marked decrease in Le^x oligosaccharide expression. Other markers such as Le^a-Le^x showed no difference in expression between Barrett's and non-Barrett's carcinomas. Details of this study are provided in ref. 7 (see appended manuscript).

Task 3. As described in the original proposal we developed methods using a digitizing image processor for estimating quantitatively the amounts of the markers described in tasks 1 and 2. Using the Quantimet 500+ Image Processing System we were able to estimate in tumor sections the fraction of cancer cells which were positive for a given fluorescent marker (above a defined base level determined from negative control cells) and independently to estimate the mean fluorescence signal per cell by integrating over a field containing a defined number of cancer cells in a tumor section. Each of these quantitative measurements was referenced to positive and negative controls obtained from sections of cloned cancer cells developed and previously studied in this laboratory. When the analyses of tasks 1 and 2 were repeated using quantitative cutoffs to define quantitatively a positive specimen, results similar to those reviewed under tasks 1 and 2 were obtained. Indeed, the results of Figures 2 and 3 (Appendix) are those of a cutoff of >0 and zero, but other cutoff ranges carried out in statistical analyses by the Biostatistics Core Laboratory of the University of Colorado Cancer Center yielded analogous results.

Task 4. This research was intended to test the most promising markers of unfavorable prognosis for their enrichment in metastatic lesions. Since none of the markers that were examined here were demonstrated to be useful prognostic markers, this task had no basis to be developed and it was therefore not pursued.

Task 5. This research was organized to identify the glycosyltransferases or other activities that are abnormally expressed in breast cancer cells that lead to aberrant expression of specific marker oligosaccharides. To this end we constructed cDNA libraries from a human lung cancer cell line (NU6-1) that overexpress Le^a-Le^x cell surface oligosaccharides and screened these libraries for long cDNA inserts that would alter the expression of Le^a-Le^x in cloned cancer cell lines that either express or do not express Le^a-Le^x. We identified 2 cDNAs corresponding to genes that strongly influence Le^a-Le^x expression. We sequenced these cDNA for comparison with the human genome data bank. One gene codes for the already known Decay Accelerating Factor (DAF) and the other that we named SIL is a previously undiscovered gene. These genes when transfected into Le^a-Le^x expressing cancer cells drastically reduce the amount of Le^a-Le^x detected on the surface of the cancer cell without affecting expression of other cell surface antigens (see Table 2 Appendix).

More recently a new cDNA library from mRNA of line NU6-1 was constructed in the vector pBK CMV. This is an expression vector for mammalian cells. Screens of this library yielded two clones that have unique sequences having partial homology to human fucosyltransferases. One is similar to human alpha (1,3) fucosyltransferase and the other to human alpha 1,3/1,4) fucosyltransferase. When transfected into human cancer cells negative for Le^a-Le^x, transfectants acquire new cell surface oligosaccharides detected by our panel of monoclonal antibodies. These cDNAs represent genes heavily expressed in Le^a-Le^x positive cancer cells and are candidate genes whose expression is required when breast cancer cells acquire the Le^a-Le^x cell surface marker. The sequences of these genes have been submitted to GENBANK.

During the course of these studies we also identified a previously unknown and potentially important level of control in regulating cell surface oligosaccharides of cancer cells. In the studies related to tasks 1 and 2 we observed significant heterogeneity in marker oligosaccharides among the cells of ductal breast carcinomas. For example, MAB 43-9F recognizing Le^a-Le^x reacts with nearly 100% of the cells of a few breast carcinomas and about 30% of the carcinomas have no positive cells, but the majority of the carcinomas have a fraction of cells that are positive, ranging from 1 to 100% of the cells in a tumor section. We are now beginning to understand some of the reasons for this heterogeneity. It was noted that cloned cell lines derived from a Le^a-Le^x positive cell could also show heterogeneity during cell culture. Studies of this phenomena showed that, in order to express Le^a-Le^x, cancer cells must be in physical contact with other Le^a-Le^x positive cells. The critical cell-cell contact is dependent on cell type. For example, in order to express Le^a-Le^x, NU6-1 cells must be in contact with other Nu6-1 cells, cells of other carcinomas will not necessarily substitute. Thus it seems that the studies of cell surface oligosaccharides can inform about the status of cell-cell signaling. Details of these experiments are described in reference # 8 (see the attached manuscript in Appendix).

Task 6. We found that the occurrence of Cathepsin D on tumor cells of the low-risk ductal carcinoma patients was not correlated with relapse frequency or survival (Appendix Table 1 and Figs 2 and 3). Likewise we could not find a statistically significant relationship between relapse free survival and either the fraction of Cathepsin D positive tumor cells or the intensity of staining the tumor cells with fluorescent anti-Cathepsin D MAB.

KEY RESEARCH ACCOMPLISHENTS

- Demonstrated that the expression of certain tumor-associated cell-surface oligosaccharides (example Le^a-Le^x) is dependent on specific cell-cell contacts among the cancer cells.
- Found that there was no statistically significant correlation between recurrence-free survival of patients having low-risk ductal breast carcinoma and the expression of any of the studied cell-surface oligosaccharides (Le^a-Le^x, Le^a, Sialyl-Le^a, Tn antigen, Sialyl-Tn, and Le^x).
- Demonstrated that esophageal adenocarcinomas arising in Barrett's epithelium are marked by decrease expression of Le^x oligosaccharides relative to the non-Barrett's adenocarcinomas.
- Found that there was no statistically significant correlation between recurrence-free survival of patients with low-risk ductal breast carcinoma and the expression of Cathepsin D.
- Showed that the cell-surface oligosaccharide Le^a-Le^x is a marker for tumors derived from embryonic endoderm.

REPORTABLE OUTCOMES

The following manuscripts and abstracts were published:

Stranahan, P., Laroe, J., Reedy, D., Rahim, I., Overland, M., and Pettijohn, D. (1994). Expression of the Carcinoma Associated Le^a-X oligosaccharide in Vertebrate Endoderm and its Fetal Derivatives. Oncology Rep. 1, 607-611.

Stranahan, P., Laroe, J., McCombs, R., Goldsmith, A., Rahim, I., Overland, M. and Pettijohn, D. (1996) Cell-cell interactions influence oligosaccharide modifications on mucins and other large glycoproteins. Glycoconjugate Journal 13, 741-747.

Engel U., McCombs, R., Strananhan, P., Pettijohn, D., and Hage, E. (1997). Decrease in Le^x expression in esophageal adenocarcinomas arising in Barrett's epithelium. Cancer

Epidemiology, Biomarkers & Prevention, 6, 245-248.

Stranahan, P., LaRoe, J., McCombs, R., Rahim, I., Kuhn, C., and Pettijohn, D. (1998). Oligosaccharide Le^a-Le^x: A cell surface marker for carcinomas derived from embryonic endoderm. Oncology Reports 5, 235-239.

Stranahan, P.L, LaRoe, J., Overland, M. and Pettijohn, D. E. Oligosaccharide Lea-X: An immunological and immuno-cytochemical marker for carcinomas derived from embryonic endoderm. (1994) Abstract - Cancer Research, December, 1994.

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Stranahan, P. L., Wahl, D., Rahim, I., and Pettijohn, D. (1999). Dexamethasone induced growth inhibition parallels in vitro decrease in NSCLC cell Le^a-Le^x expression. Abstract. AACR Annual Meeting. Proc. of AACR. Vol. 40, 1999.

CONCLUSIONS

The final results of this project did **not** support our earlier findings with animal model systems indicating that more aggressive carcinomas were those having tumor cells marked with cell surface Le^a-Le^x oligosaccharides. Rather the studies summarized here showed that patients having low-risk ductal breast carcinomas which were positive for Le^a-Le^x had a post-surgical prognosis that did not significantly differ from similar patients with Le^a-Le^x negative tumors. A panel of related cell-surface oligosaccharides, which are variably expressed in different ductal breast carcinomas, were also studied to define their potential as prognostic markers in low-risk ductal breast carcinomas. The findings indicated that oligosaccharides, Le^a, T-antigen, Tn-antigen, sialyl- Le^a or Le^x showed no significant correlation with prognosis.

Results from this project developed specific cell surface oligosaccharides as useful tumor-associated markers. It was demonstrated that Le^a-Le^x is a cell-surface marker for tumors derived from embryonic endoderm. It was also shown that esophageal adenocarcinomas arising in Barrett's epithelium are marked with Le^x differently from non-Barrett's carcinomas. Two new cDNAs were cloned and sequenced coding for what are apparently new human fucosyltransferases that are overexpressed in cancer cells making Le^a-Le^x. These are candidates for genes that are usually expressed in breast cancer cells of certain low-risk ductal carcinomas. Finally, two genes (SIL and DAF) were identified and sequenced that regulate the expression of cell-surface Le^a-Le^x in transfected cancer cells. In future research these developments could provide useful tools for tumor analysis and diagnosis. .

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6. Stranahan, P., Andersen, J., Archer, P., McCombs, R., and Pettijohn. D. (1997). Cancer associated Le^a-Le^x is predictive for increased risk of disease relapse in low-risk breast cancer. Abstract. AACR Annual Meeting. Proc. of AACR. Vol.38, 1997.

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BIBLIOGRAPHY AND LIST OF PERSONNEL

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APPENDIX

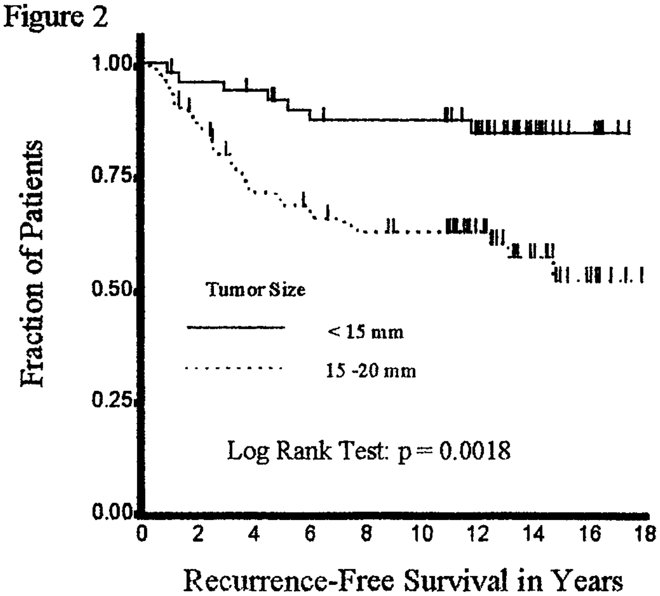
Table 1 shows results from the analysis when epitope is treated as continuous variable. Tumor size is the only significant factor among the covariates investigated. Figure 2 shows the Kaplan-Meier estimate of the tumor size effect.

Table 1

Covariate	P	Effect direction
Age	0.6449	The older, the worse prognosis
Menopausal	(0.4150)	pre-menopausal women have better prognosis
Tumor Size	**0.0013**	The smaller the tumor, the better the prognosis
Grade	0.5284	The higher the grade, the worse the prognosis
LeA-LeX	0.5970	The larger the percent of expression, the worse the prognosis
LeA	0.8850	The larger the percent of expression, the worse the prognosis
LeX	0.1340	The larger the percent of expression, the better the prognosis
Sia-LeA	0.6157	The larger the percent of expression, the better the prognosis
S.Tn	0.8921	The larger the percent of expression, the worse the prognosis
Tx	0.2021	The larger the percent of expression, the better the prognosis
Tn	0.9931	The larger the percent of expression, the worse the prognosis
Cath	0.6498	The larger the percent of expression, the worse the prognosis
ER	0.6887	No obvious pattern
PG	0.2910	No obvious pattern

- P-value for Log Rank Test is in parenthesis.

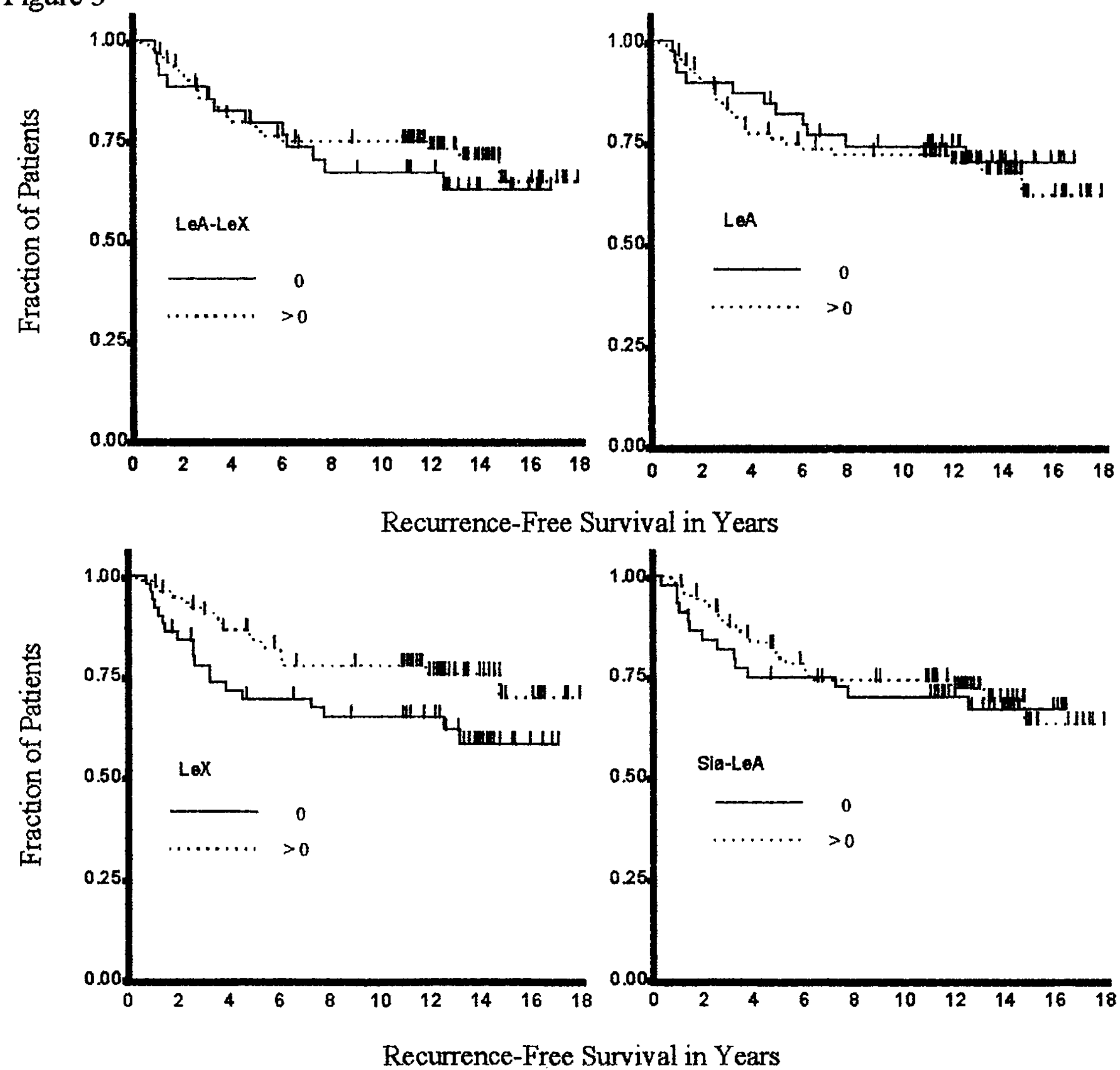
Figure 2

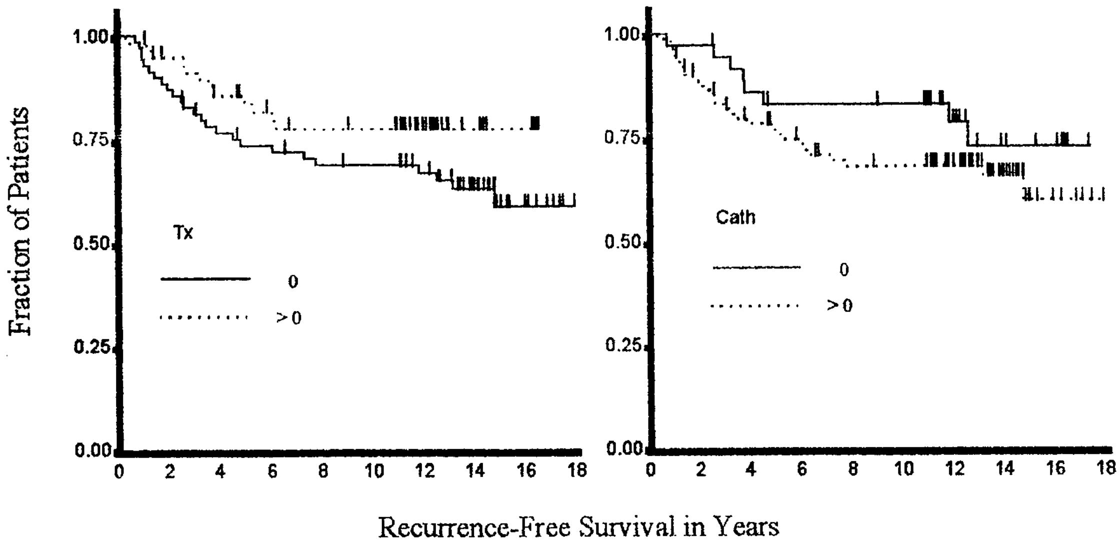


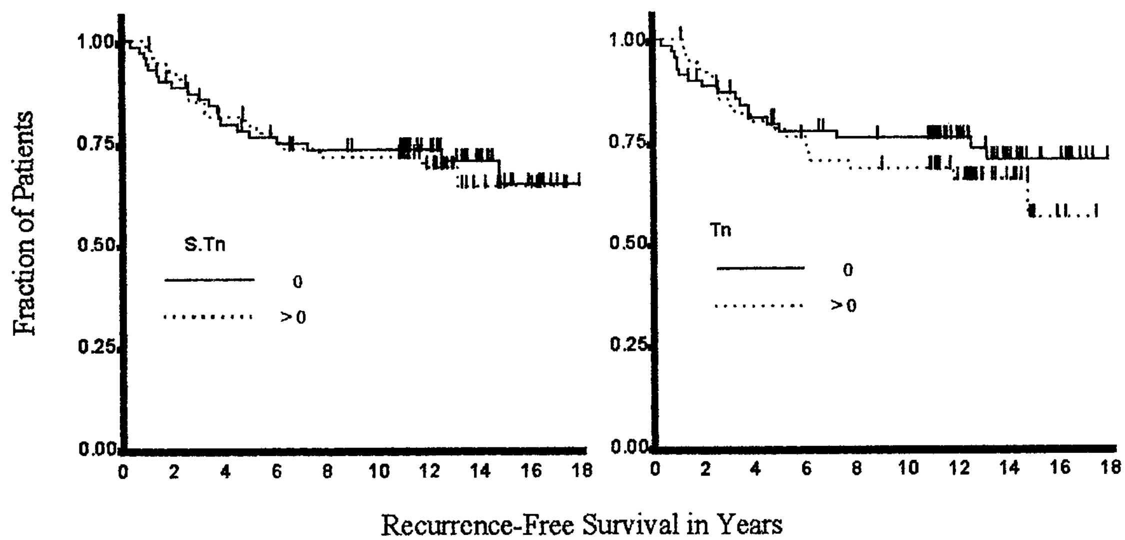
In order to use all of 126 observations in the analysis, Epitope variables are dichotomized and Kaplan-Meier model is used to look at the effect of each epitope on recurrence free survival. Table 2 shows results from the analyses when epitope is dichotomized. Again, none of them is statistically significant. To visualize the results, Kaplan-Meier estimates are ploted(Figure 3)

Covariate	P
Age	0.6449
LeA-LeX	0.5744
LeA	0.6326
LeX	0.0813
Sia-LeA	0.7113
S.Tn	0.8276
Tx	0.1376
Tn	0.4140
Cath	0.2053

Figure 3







III Multivariate Cox Regression Analysis

Using step-down selection strategy and 0.05 as the significance level for a covariate to leave the model, a multivariate Cox regression were performed. Candidate covariates include: `ll_inf lea_inf lex_inf sl_inf stn_inf tx_inf cath_inf age tmr_size grade and meno.`

78 patients are used in this analysis with 26 recurrence events and 52 censored records. It turns out that tumor size is the only significant factor left in the final model. The next most significant factors are LeX with p-value equal to 0.1141 after adjusting for tumor size.

TABLE 2

% of Cells Positive for Specific Monoclonal Antibodies Before and After Transformation by SIL or DAF

Cell Lines	Mab 43-9F	Mab CO514	Mab P12	Mab 19-9	Mab IE3	Mab TKH2	Mab HH8
NU-6--1	50%	30%	Neg	85%	Neg	Neg	Neg
NU-6-1-SIL	1%	50%	30%	90%	10%	Neg	Neg
NU-6-1-DAF	10%	10%	Neg	2%	Neg	Neg	Neg
NE-18	Neg	Neg	35%	Neg	10%	Neg	Neg
NE-18-SIL	Neg	Neg	60%	Neg	Neg	Neg	Neg
NE-18-DAF	Neg	Neg	Neg	Neg	Neg	Neg	Neg
HT-29	10%	50%	2%	50%	Neg	Neg	Neg
HT-29_SIL	80%	80%	10%	80%	Neg	Neg	Neg
HT-29-DAF	80%	80%	2%	80%	Neg	Neg	Neg
T47D	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T47D-SIL	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T47D-DAF	Neg	Neg	Neg	Neg	Neg	Neg	Neg
COS-1	50%	Neg	Neg	Neg	Neg	Neg	Neg
COS-1-SIL	80%	Neg	Neg	Neg	Neg	Neg	Neg
COS-1-DAF	80%	Neg	Neg	Neg	Neg	Neg	Neg
H157	Neg	Neg	Neg	Neg	Neg	Neg	Neg
H157-SIL	Neg	Neg	Neg	Neg	Neg	Neg	Neg
H157-DAF	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Oligosaccharide Le^a-Le^x: A cell surface marker for carcinomas derived from embryonic endoderm

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Abstract. An immunohistochemical evaluation of Le^a-Le^x expression by adenocarcinomas of the biliary tree, pancreas, colon and stomach was undertaken as examples of epithelial tumors derived from embryonic endoderm. This complements previous studies showing that Le^a-Le^x was present on the cell surface of non-small cell lung carcinomas, some non-lung carcinomas, and is a prognostic marker for squamous cell lung carcinomas. All of the tumor specimen evaluated were positive and no expression of Le^a-Le^x was detected in derivatives of neural, connective or muscle tissues. These findings indicate that it could be informative to examine the biological significance of Le^a-Le^x not only in carcinogenesis but during embryogenesis, as well.

Introduction

The blood group related carbohydrates A, B, H and the Lewis antigens are developmentally regulated. They have been implicated directly or as modulators of cell-cell adhesion and have also been found to be expressed aberrantly in certain cancers (1,2). In the fetal stage the appropriate epitopes for the blood type are expressed, whereas in cancers altered expression by the transformed epithelial cells occurs. These alterations can include a re-expression of the fetal epitopes, a deletion of the the antigen that should be expressed normally, or the expression of an antigen that is incompatible with the blood type of the individual (3,4).

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Abbreviations: Mab, monoclonal antibody; FITC, fluorescein isothiocyanate

Key words: Le^a-Le^x, oligosaccharides, biomarkers, mucin

A number of anti-Le antibodies have been evaluated as probes for cancer markers (5-9). Human gastric cancers show an enhanced expression of Le^a and loss of ABH (10). Distal colon cancers express Le^b and Le^y (11). Sialyl-dimeric Le^x, (SLX) an oncodevelopmental carbohydrate antigen has been shown to be expressed in human colorectal carcinomas, both on glycolipids and on mucin proteins. The cells expressing high concentrations of sialyl Le^x show an increased invasive capacity *in vitro* versus the low expressors (12). Long and short chain Le^x antigens are significantly enhanced in colonic carcinoma (13). A study of an isolated population in the Colombian Andes that showed a high incidence of gastric cancer, revealed abnormal expression of Le^a in gastric epithelium (14). It has been postulated that several discrete cell populations at different stages of progression of tumors show variable patterns of glycosylation. Moreover, a single tumor can show mosaicism in the expression of carbohydrate antigens within it (15). Changes in expression of Lewis antigens have been observed in human intestinal metaplasia, gastric adenoma and gastric carcinoma as well as adenocarcinoma associated with Barrett's esophagus (4,16). Lewis epitope modulation has also been demonstrated *in vitro*, secondary to changes in cell density (17). An increased frequency of erythrocyte Lewis negative individuals was found in colon and urinary bladder cancer patients, while four out of eight patients were shown to have α1-4fucosyltransferase in their saliva and appeared to have converted from Lewis positive to Lewis negative phenotype as their cancer advanced (18).

Type 1 oligosaccharide backbones (Galß-3GlcNAcß1-3Gal) give rise to the Le^a, Le^b and H antigens and type 2 backbones (Galß1-4GlcNAcß1-3Gal) yield Le^x, Le^y and H antigens. An extended Lewis antigen, Le^a-Le^x, was found to be expressed on the surface of a cell line derived from a human squamous lung carcinoma (19,20). This previously unreported oligosaccharide epitope, having the structure Galß1-3[Fucα1-4]GlcNAcß1-3Galß1-4[Fucα1-3]GlcNAcß1-3Galß1-4Glc was identified and shown to bind with high affinity to Mab 43-9F and appears to be confined to large glycoproteins and may not be associated with glycolipids (19-21). Le^a-Le^x is expressed by human squamous lung carcinomas and adenocarcinomas but not by small cell carcinomas of the lung. The appearance of this epitope was investigated in several variants of a squamous lung carcinoma

(SLC-L11) cell line. The Le^a-Le^x positive variants grow, and are invasive when placed subcutaneously or intrabronchially into nude mice and rats, while those clones which do not express the Le^a-Le^x oligosaccharide show regression after initial growth (20,22).

The extended Le^a-Le^x also has a precisely controlled pattern of expression during embryogenesis. A survey of representative vertebrate embryos and fetuses in various stages showed that the embryonic and fetal cells which express the epitope are derived from embryonic endoderm (23). Adult tissues derived from mesoderm and ectoderm do not express this epitope. The expression of this oligosaccharide is rare in normal epithelials as compared to its upregulation in non-small cell lung carcinoma cells (24). It has also been demonstrated that Le^a-Le^x is a sensitive marker of carcinoma of human testes and that the presence or absence of this oligosaccharide epitope also has significant prognostic value in human squamous lung carcinomas (25,26).

The present study is a survey of this epitope in various carcinomas derived from the epithelials that rarely express the epitope in mature adult tissues.

Materials and methods

Tumor specimen. Twenty cases, each, of cholangiocarcinoma, pancreatic adenocarcinoma, gastric adenocarcinoma and adenocarcinoma of the colon which had been previously paraffin embedded were procured from the University of Colorado Health Sciences Center surgical pathology archives. All adenocarcinomas were surgical specimen and included both male and female patients. From each surgical specimen 5-10 blocks were taken which represented the resection lines, non-tumorous mucosa and pathologic mucosa. If metaplastic and dysplastic epithelium was present those blocks were chosen, as well. Tumor differentiation in all groups included poorly, moderately and well differentiated adenocarcinomas.

Paraffin sections were taken from the original blocks. The tissue was de-paraffinized and prepared for immunohistochemistry by vigorously washing three times, ten minutes each, in phosphate buffered saline and soaking overnight in phosphate buffered saline.

Antibody. Mab 43-9F (IgM) which recognizes the oligosaccharide epitope Le^a-Le^x was purified from serum-free culture medium (RPMI 1640) of the 43-9F hybridoma as previously described (20). In some experiments the antibody containing media was used without purification. Purified 43-9F Mab was applied at a concentration of about 2 µg/ml (diluted in PBS), while 43-9F in serum-free culture media was applied after dilution of 1:40 (in PBS). Each tissue section was incubated with primary antibody for one hour in humidity chambers at room temperature. After one hour, the sections were thoroughly washed three times in room temperature phosphate buffered saline. FITC secondary IgM antibody [(goat anti-mouse) Sigma, USA] was applied at a concentration of about 2 µg/ml diluted in phosphate buffered saline and the sections were incubated for one hour in humidity chambers at room temperature. Again, sections were thoroughly washed after incubation and several drops of antifade coverslip mounting media were applied to the sections and the slides were coverslipped.

On a routine basis, hematoxylin and eosin (H&E) sections were obtained adjacent to those sections which were evaluated by immunofluorescence.

Evaluation. Evaluation of the patient specimen was on the basis of the intensity of the reaction product (0-4 plus) and on the percentage of tumor cells which fluoresced (0-100%). Semi-quantitatively, each specimen was placed into one of the following groups: <5%, 6-10% positive cells, 11-25% positive cells, 26-50%, 51-75% positive cells and >75% positivity. The recording of positive tumor cells was based on all the investigated tumor tissue, estimating the fraction and counting of 5-10 high power fields (HPF). With each experiment a known positive carcinoma control as well as a negative control was processed. Also, non-specific reactivity was evaluated by paralleling a section with PBS replacing primary antibody.

The tissues were stored at 4˚C after initial evaluation. Re-evaluation of the tissues was possible for up to two months at which time the reaction product faded dramatically. Photomicrographs were taken on a Leitz Dialux 20 microscope with UFX-II photographic attachment. Filter cube N for FITC incident light microscopy was employed.

Statistical analysis. Statistical evaluation of the data was performed using the ANOVA multivariant test. A two-tailed analysis was carried out. Only results with $P<0.001$ were regarded as significant. These analyses were performed in the Department of Mathematics and Computer Sciences at Metropolitan State College of Denver.

Results

Adenocarcinomas derived from the pancreas, colon, stomach and bile ductules were studied as examples of epithelial derived tumors arising from embryonic endoderm. The oligosaccharide Le^a-Le^x is expressed in 100% of the adenocarcinomas studied while associated dysplastic/metaplastic and normal tissues expressed this epitope much less frequently (Table I and Fig. 1). Characteristic patterns present in moderately differentiated adenocarcinomas from pancreas, colon and bile ductules are shown in Figs. 2-4. The malignant epithelial cells of the twenty cases of each organ evaluated ranged from poorly differentiated to well differentiated carcinomas. The histologic pattern (poorly differentiated to well differentiated) did not alter the malignant epithelial cells' ability to express Le^a-Le^x ($P>0.05$). Some of the tumors contained cells which only formed rare glands while others formed glands which had an almost normal appearance with normal apical-basolateral cellular orientation. These malignant cells express Le^a-Le^x at the apical surfaces as well as within the cytoplasm of the cellular elements. Many fields were observed in which intralumina secretory material gave a 4+ positivity for the presence of the oligosaccharide (Fig. 3). No connective tissue cells, muscle cells or nerve fibers were observed to express Le^a-Le^x.

The intensity of cellular staining for each case was quite consistent. Cells of these adenocarcinomas did not show

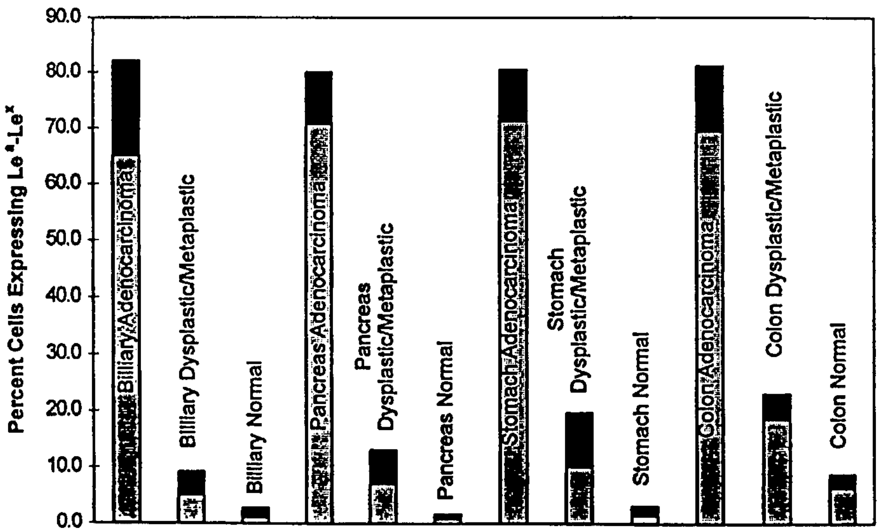

Figure 1. Le^{a}-Le^{x} expression by adenocarcinoma, atypical and normal epithelial cells arising in selected derivatives of embryonic endoderm. Darker regions atop bars represent standard deviations. Lighter regions display means.

Table I. Le^{a}-Le^{x} oligosaccharide expression in selected carcinomas derived from embryonic endoderm.

Tumor site	Cases	Cases expressing epitope	Percent cells expressing epitope
Biliary tree	20 total		
Adenocarcinoma		20/20	25-100
Dysplastic/metaplastic		19/20	0-10
Normal		10/20	0-5
Pancreas	20 total		
Adenocarcinoma		20/20	25-100
Dysplastic/metaplastic		20/20	0-20
Normal		11/20	0-5
Stomach	20 total		
Adenocarcinoma		20/20	25-100
Dysplastic/metaplastic		20/20	0-25
Normal		16/20	0-10
Colon	20 total		
Adenocarcimona		20/20	25-100
Dysplastic/metaplastic		20/20	10-50
Normal		20/20	0-10

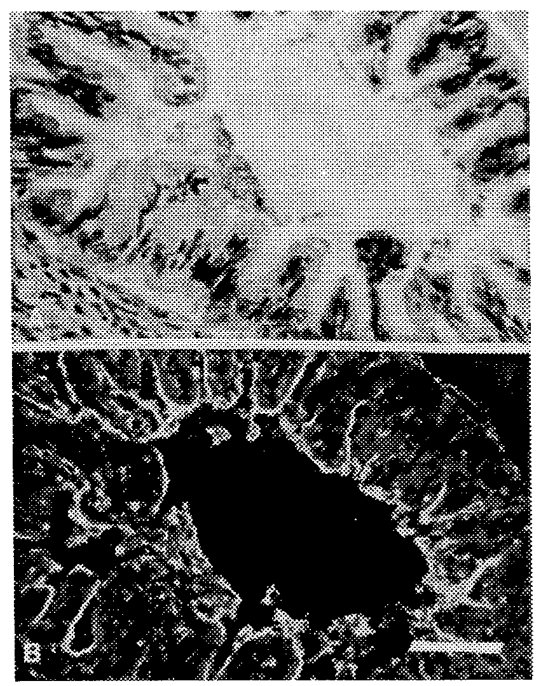

Figure 2. A, Hematoxylin and eosin section revealing malignant epithelial cells forming glands in a moderately differentiated cholangiocarcinoma. B, FITC immunofluorescent microscopy of a section adjacent to that photographed in (A) using Mab 43-9F. Approximately 75% of the malignant epithelial cells express oligosaccharide Le^{a}-Le^{x}. Original magnifications x200. Bar = 150 μ.

partial positivity and were either negative or brilliantly positive with the concentrations of primary and secondary antibodies applied. Also, the percentages of cells expressing Le^{a}-Le^{x} in each case was remarkably high. Tumors were almost completely positive with Mab 43-9F with only an occasional tumor showing 50% malignant cells (Fig. 1 and Table I).

Normal epithelial cells associated with these neoplastic cells in each organ express this oligosaccharide much less frequently. In each of the present cases adjacent normal,

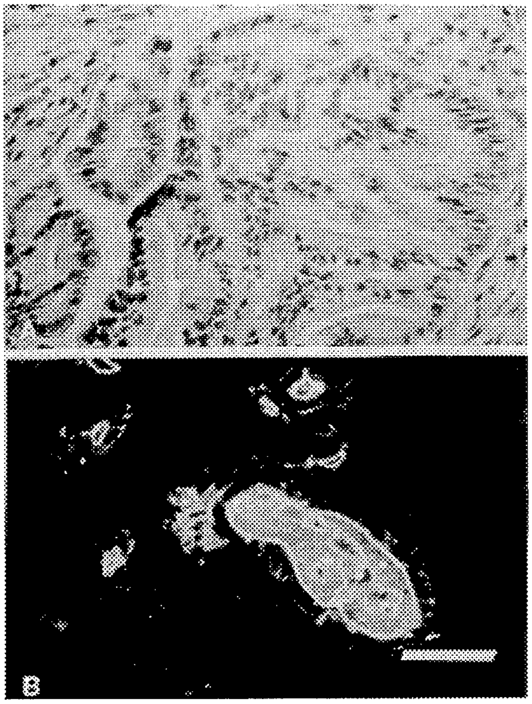

Figure 3. A, Hematoxylin and eosin section revealing irregular glands of a moderately differentiated adenocarcinoma of the pancreas. B, FITC immunofluorescent microscopy of a section adjacent to that photographed in (A) using Mab 43-9F. Approximately 75% of the malignant epithelial cells of this tumor express the Le^a-Le^x epitope. Original magnifications x200. Bar = 150 μ.

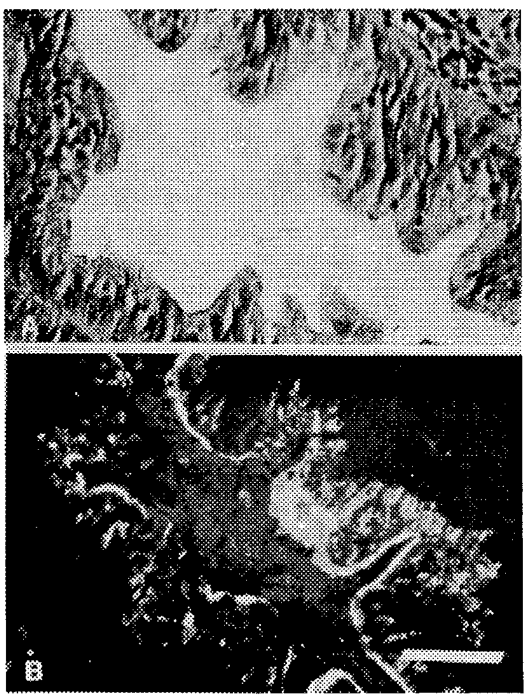

Figure 4. A, Hematoxylin and eosin section revealing malignant glands composed of irregular epithelial cells in a moderately differentiated adenocarcinoma of the colon. B, Fluorescent microscopy of a section adjacent to that photographed in (A) using Mab 43-9F. Approximately 90% of the malignant epithelial cells express Le^a-Le^x. Original magnifications x200. Bar = 150 μ.

metaplastic and/or dysplastic cells were evaluated if tissue with such cells was available. Less than 10% of normal epithelial cells were positive for Le^a-Le^x. As the level of cellular atypia increased, the expression of the epitope increased (Table I and Fig. 1). In no case did expression within normal, metaplastic or dysplastic epithelium approximate expression within tumor ($P<0.001$) (Fig. 1, Table I).

Discussion

Previous studies of the Le^a-Le^x epitope using Mab 43-9F demonstrated that expression in normal adult human tissues and in cancer cell lines grown in tissue culture was primarily associated with large glycoproteins and especially mucins (19,20). Preliminary findings suggested that cells expressing the epitope are derivatives of the embryonic endoderm (23).

Initial studies using Mab 43-9F showed the epitope Le^a-Le^x was associated with squamous cell carcinoma of the lung. It was also found to be secreted on associated mucins by the squamous lung carcinoma (SLC-L11) cell line (20,22). The present results extend the previous findings to other carcinomas which are derived from embryonic endoderm and show that oligosaccharide Le^a-Le^x is also up-regulated in adenocarcinomas derived from pancreas, colon, stomach, and bile ductules. Immunostaining with Mab 43-9F suggests that Le^a-Le^x is primarily distributed on the apical surfaces of the endodermal cells or associated with apical extracellular material. This is also the case in rare differentiated normal adult cells. However, tumor cells seem to lose the ability to differentiate apical surface expression and the epitope is present at the basolateral surface as well. This suggests that trafficking of glycoproteins could be aberrant in tumor cells expressing the Le^a-Le^x epitope. Whether other Lewis epitopes are thus altered is presently not known.

There is minimal expression of this extended Lewis antigen (Le^a-Le^x) by normal gall bladder, pancreatic, colon, and stomach columnar epithelial cells. The Le^a-Le^x up-regulation upon malignant transformation may be similar to that of other Lewis antigens as reported in human gastric carcinomas (7).

It was postulated that oligosaccharide Le^a-Le^x is normally involved in cell-cell/cell-ECM signaling and/or communication (19,23). The postulated disruption of the normal cell-cell interactions could be a facet of the autonomous growth and tissue invasion associated with malignant transformation.

Normal Le^a-Le^x oligosaccharide expression in the embryonic stage has been shown to be temporally regulated, as might be anticipated if it were involved in cell-cell communication (23). Also, cells growing *in vitro* at different cell densities have altered expression of this epitope (17). Malignant transformation appears to be co-ordinated with expression of this epitope at an inappropriate phase of the organismal life cycle (23).

Oligosaccharide Le^a-Le^x may be a useful diagnostic and prognostic marker in caring for patients with tumors derived from tissues of endodermal origin. Further studies involving the cell surface glycoconjugates expressing Le^a-Le^x could enhance our understanding of mechanisms involved in malignant transformation and tumor invasion.

Acknowledgements

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Cell–cell interactions influence oligosaccharide modifications on mucins and other large glycoproteins

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Intratumoral phenotypic diversity is well documented with regard to tumor associated carbohydrate antigens (TACA). The factors which control the expression of these cell-surface oligosaccharides on different cells of the same tumor are not understood. We investigated the expression of a panel of mucin associated oligosaccharides in cell lines growing at different surface densities (number of cells per cm^2 of growth flask). Results show that the apparent expression of extended Le^a-Le^x, Le^a and Le^x, sialyl Le^a, Tn and sialyl Tn varies with density of growth by an invasive human squamous cell lung carcinoma cell line (NU6-1), a benign variant (NE-18) and the human lung epithelial cell line BEAS-2B. The results indicate that one of the factors influencing the apparent expression of mucin-associated oligosaccharides is cell-cell interactions.

Keywords: mucins, oligosaccharides, cell-cell interactions cancer, aberrant expression

Abbreviations: Mab, monoclonal antibody; FIT, fluorescein isothiocyanate; TACA, tumor associated carbohydrate antigen

Introduction

The factors controlling the elaboration of complex oligosaccharide sequences on mucins and other large glycoproteins are poorly understood [1–4]. The oligosaccharides of these large glycoproteins are primarily expressed on the cell surface or are part of complexes appearing in the extracellular environment [5–7]. It has often been observed that adjacent cells in normal tissue that are indistinguishable by other criteria can differ in the expression of specific cell surface oligosaccharides. Moreover, nearby cells in the same tumor often vary in their cell surface oligosaccharides detected with specific monoclonal antibodies (Mabs) [8, 9]. Cloned cells derived from such tumors can be selected for homogeneity of oligosaccharide expresssion, but the expression is often unstable, leading rapidly to divergent expression in different cells of the same clone [10]. These observations, while not easily explained, lead one to consider the possibility that environmental triggers acting extracellularly may effect differential responses in genetically identical cells.

Studies in this laboratory have focused on aberrant glycosylation of mucins and other large glycoproteins in squamous cell lung cancer and cancers of the gastrointestinal tract [11–13]. Initially we described expression of a previously unidentified extended Lewis antigen (Le^a-Le^X) in squamous cell lung carcinoma which is recognized by Mab 43-9F. Le^a-Le^x is known to be exclusively associated with glycoproteins in contrast to other Lewis antigens which are glycoproteins/glycolipid carbohydrate moieties [14]. These studies were extended to include the use of additional Mabs to identify expression of other Lewis antigens which have each, individually, been described as tumor associated carbohydrate antigens (TACA) by others [15–19]. Utilization of a panel of

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biomarkers on patient specimen was initiated in each case in order to increase the probability of concordant aberrant glycosylation which would lead to patterns that might have diagnostic and/or prognostic significance. Further, emerging patterns could alter patient management decisions.

The present study characterizes previously unrecognized cellular diversity of human squamous cell lung carcinoma cells. We demonstrate here that oligosaccharide expression by these variants appears to be regulated by cell-cell interactions. These findings suggest that any experimental strategy for defining prognosis based on cell surface oligosaccharides should take into account the potential capacity for intracellular mosaicism.

Methods

Cell lines

Two previously described clones of the human squamous lung carcinoma line RG-SLC-L11 and the SV40 transformed human lung epithelial cell line BEAS-2B were removed from liquid nitrogen and were allowed to attach to plastic vessels, using RPMI 1640 medium plus 15% fetal bovine serum (clone NE-18 and BEAS-2B) or bovine calf serum supplemented with iron (clone NU6-1) as previously reported [8]. Single cell suspensions were prepared from confluent monolayers by a 5–10 min incubation at room temperature in 0.05% trypsin plus 2 mM EDTA or in 2 mM EDTA alone as previously described [8]. None of the various trypsin or EDTA concentrations or the supplementation with calf serum affected the previously described tumorigenicity [20]. Single cell preparations were prepared for examination by allowing these diluted cells to attach on eight well, teflon coated slides (Cell-Line). These sparse single cells were allowed to grow for 24 h. They were then fixed in 5% phosphate buffered formalin, permeabilized in acetone, at 4 °C for 5 min and stored at 4° in PBS until use. Small colonies were established after single cell plating by allowing growth until an average of 25 cells, each, were counted in 100 colonies. At this point, the eight well slides were washed and treated as the single cell slides. Other cultures were allowed to grow to confluence or post-confluence (2–5 days after confluence). At the appropriate cell density, the slides were washed, fixed and permeabilized as previously discussed [20].

Monoclonal antibodies

Six Mabs specific for different oligosaccharides were used. Anti Le^a-Le^x (43-9F), and anti Le^a (CO-514) were purified from serum-free culture medium of the respective hybridomas as previously described [11, 21]. In some experiments the antibody-containing media was used without purification. Anti-Le^x (P12), anti-Tn (IE3) and anti-sialyl Tn (TKH2) were received as antibody-containing media. Anti sialyl Le^a (Mab 19-9) was received in purified form. The monoclonal antibodies were applied to plated, fixed cells, as previously reported [11, 21].

Immunohistochemistry and evaluation

Each cell specimen was incubated with primary antibody for 1 h in humidity chambers at room temperature, washed, incubated with FITC secondary polyvalent IgG/IgM antibody (goat anti-mouse) and examined using a Leitz Dialux microscope, as previously described [11].

Evaluation of the preparations was on the basis of the intensity of the reaction product (0–4+) and on the percentage of tumor cells which fluoresced (0–100%). With each experiment a known positive carcinoma control as well as a negative control was processed. Also, non-specific reactivity was evaluated by paralleling fixed, permeabilized cells with primary antibody omitted.

Purification of mucins and large glycoproteins

NU6-1 cells were grown to near confluence in plastic flasks in RPMI 1640 media plus 5% bovine calf serum (BCS) supplemented with iron. The medium was removed, the attached cells were washed repeatedly with media lacking BCS, and then grown for 3 days in the serum-free RPMI media containing 2 $\mu Ci\,ml^{-1}$ [^3H] glucosamine (2.0 $Ci\,mmol^{-1}$). The spent media was recovered, mixed with identical media from unlabeled cells, centrifuged to eliminate any debris, glycoconjugates precipitated with ammonium sulfate, the precipitate gently solubilized in PBS containing 0.1% Tween + 0.5 mM PMSF, and the glycoconjugates were applied to a Sephacryl S500 column equilibrated with PBS/Tween solution and fractions collected while applying fresh PBS/Tween solution.

Analysis of epitope expression on purified mucins

Utilizing the Bio-rad immunoblot apparatus, 10 μl of each fraction collected off the Sephacryl S500 column was absorbed and washed on appropriately prepared Immubilon, as previously described [11]. The Imobilon was blocked with competing proteins and incubated with Mab 43-9F for 1 h at room temperature as described. After washing three times with PBS the sheet was incubated with ^{125}I labeled IgM secondary antibody, washed, dried and reaction product measured as previously described [11].

Incubation of NE-18 cells (epitope negative) with concentrated media from NU6-1 cells (epitope positive)

The epitope negative cell line NE-18 was grown on eight well teflon coated slides to near confluency in 15% FCS supplemented RPMI media. Mucins and other large glycoproteins were concentrated from previously harvested media of NU6-1 cells by ammonium sulfate precipitation; solubilized, dialysed and added at different concentrations to the growing NE-18 cells. Growth of cells was allowed to continue for another 24 h. The cells were then stained with oligosaccharides specific Mabs and evaluated as described above.

Results

Three different human cell lines, BEAS-2B, NU6-1 and NE-18, growing as separate cells, in small 25 cell colonies, confluent, or hyperconfluent were stained with six different Mabs specific for different mucin associated oligosaccharides. The term hyperconfluent is used here to denote a distribution of cells resulting from growth in three-dimension so that growing cells pile up on each other. The stained cells on individual eight well slides were evaluated independently by three individuals at different times and the results were scored independently (Table 1).

BEAS-2B cells, which are SV40 transformed normal pulmonary epithelial cells, did not express any of the six mucin associated antigens as single cells. Five of the epitopes continued to be undetectable in small colonies and confluent as well as post-confluent cultures. The sixth antigen, sialylated Le^a, was first detected in cells of small colonies. Fifty per cent of the small colony cells express this epitope in a cytoplasmic membrane bound pattern. When the cultures became confluent 100% of the cells express the epitope. In the hyperconfluent state only 5% of the cells are expressors.

The SLC-11 variant cell line, NE-18, which has been shown to regress both subcutaneously and orthotopically in nude mice, did not express five of the epitopes as single cells or as small colonies (Table 1). With confluence, 5% of the cells express Tn as detected by monoclonal antibody IE3 (Fig. 1). Le^x was expressed by single cells, cells of small colonies as well as confluent and hyperconfluent cells; however, the percentage of cells expressing varied at these different cell densities. The

Table 1. Three variant human lung epithelial cell lines were placed on eight well slides and allowed to plate down and grow to varying cell densities. Expression of each of six mucin associated oligosaccharide epitopes is shown. Variability in expression differed with cell density. Each of the cell lines was plated as single cells, grown into small colonies, grown to logarithmic confluence or allowed to continue past logarithmic phase (hyperconfluence). Oligosaccharide expression by BEAS-2B, NE-18 and NU6-1 cell lines.

	Le^a-Le^x	*Le^a*	*Le^x*	*Sialyl Le^a*	*Tn*	*Sialyl Tn*
BEAS-2B						
Single	–	–	–	–	–	–
Small colonies	–	–	–	4+/50% membrane	–	–
Confluent	–	–	–	4+/100% membrane	–	–
Hyper-confluent	–	–	–	4+/5% cell[a]	–	–
NE-18						
Single	–	–	4+/35% perinuclear	–	–	–
Small colonies	–	–	4+/35% perinuclear	–	–	–
Confluent	–	–	4+/35% perinuclear	–	4+/5% cell	–
Hyper-confluent	–	–	4+/5% perinuclear	–	4+/5% cell	–
NU6-1						
Single	4+/1% cell	4+/1% cell	4+/1% cell	4+/100% cell	– –	– –
Small colonies	4+/50% cell	4+/25% cell	4+/100% membrane	4+/100% cell	4+/30% cell	4+/30% cell
Confluent	4+/50% plates[b]	4+/35% plates	4+/50% membrane	4+/50% cell	4+/10% cell	–
Hyper-confluent	4+/100% no plates, cell only	4+/75% cell	4+/35% cell	4+/100% cell	–	4+/30% cell

[a]cell, denotes fluorescence throughout; including membrane and cytoplasmic structures.
[b]plates, epitope expression associated with angular material overlying individual cells.
–, denotes lack of epitope expression utilizing Mabs specific for each antigen.

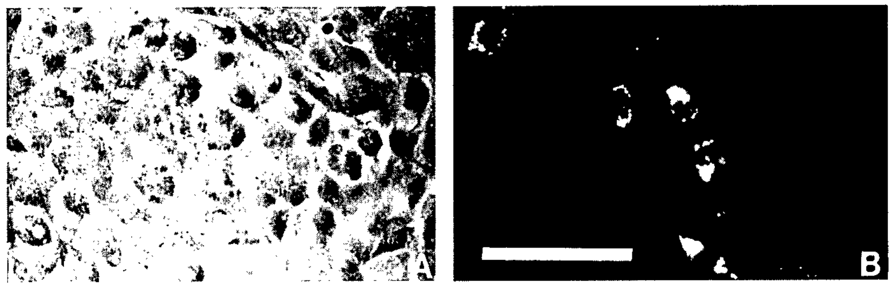

Figure 1. Tn expression by Ne-18 cells grown to confluence. Tn expression was observed by 10% of these cells at confluence. Prior to confluence no Tn expression was apparent utilizing Mab IE3 by single cells or cells in small colonies. (A. Phase microscopy; and B. FITC immunofluorescent microscopy of the same cells.) Bar, 150 μm, $\times$ 200.

single cells, cells in small colonies, and confluent cells expressed the epitope in greater concentration than did cells at hyperconfluency (Table 1).

The malignant SLC-11 cell line variant, NU6-1, showed the most variability with cell density (Table 1 and Fig. 2). Single cells expressed four of the epitopes in varying concentrations, but did not express Tn or sialylated Tn. Detection of Le^a-Le^x, Le^a and Le^x on 1 in every 100 cells was observed. One hundred per cent of single cells express sialylated Le^a (Fig. 2H). The subsequent cells of small colonies all expressed these epitopes to a certain degree. The Le^a-Le^x, Le^a, sialylated Tn and Tn oligosaccharides were expressed by only a fraction of the cells in small colonies and the expressing cells were primarily those on the periphery on the colonies (see Fig. 2C,D). By contrast the Le^x and sialylated Le^a oligosaccharides were expressed in 100% of the cells in the small colonies (details not shown). When the colonies grow to the point where cells become confluent, the cells become overlaid with a mucin gel, which, as previously reported [8] is stained by Mabs specific for Le^a-Le^x, Le^a and Le^x in a mosaic pattern which appears in geometric-like plates (Fig. 2E,F). In this case 35–50% of the cells are associated with gels that strongly express the later oligosaccharides. These confluent cells also expressed the sialylated Le^a and sialylated Tn oligosaccharides, but did not preferentially stain the mucin gel and geometric plates were not appreciated. Tn was not expressed by these cells at confluency. When the confluent cells were left to grow and pile upon each other (hyperconfluency), 100% Le^a-Le^x expressivity occurred. However the geometric plate formation is lost (Fig. 2G). In the hyperconfluent state Tn expression is also lost (Table 1).

The observed expression of new oligosaccharide epitopes as single-cells divide to form small colonies is expected to be reliably detected by the monoclonal antibodies employed in this study. However, there is less certainty that the extinction of other previously expressed epitopes would be detected as reliably by the same methods as cells approach confluence. It seems possible that the later oligosaccharide epitopes are actually expressed, but are not available for interaction with antibodies, because of specific adhesion functions in the glycocalyx or with neighboring cells. For this reason we have begun studies of glycoproteins isolated from the different cell types or their media to define the apparent extinction in molecules that have no interactions with cells or glycocalyx. One example of this analysis is shown in Fig. 3.

Soluble mucins and other large glycoproteins from NU6-1 cells were fractionated on a Sephacryl S500 column and analysed for their associated oligosaccharides using immunoblot assays and the Mabs described above. As shown in Fig. 3, glycoconjugates fractionating at positions equivalent to $M_r > 107$ and having properties of mucins [11] are associated with Le^a-Le^x. Other smaller, but also large, glycoconjugates in the M_r range 100 000 to 500 000 are also associated with Le^a-Le^x. Similar analyses using Mabs recognizing the other oligosaccharides also showed that these oligosaccharides are associated with glycoconjugates of comparable size (results not shown). Moreover, a similar analysis of mucins from NE-18 cells (showing extinct expression of Le^a-Le^x) showed no expression of Le^a-Le^x on isolated mucins (results not shown). Thus, at least in this case, there was a good correlation between extinction of an oligosaccharide epitope on confluent cells and in isolated mucins.

The above results show that the expression of different cell-surface oligosaccharides, associated with mucins and other high molecular weight glycoconjugates, varies depending on the growth density of the different types of cells. One cell line, NU6-1 expresses certain oligosaccharides like Le^a-Le^x or Le^a only on rare cells, when the cells grow without neighbors, but the fraction of expressing cells greatly increases when cells grow in colonies or in confluent sheets. However, most of the NU6-1 cells express the sialyl Le^a oligosaccharide when they are growing in single cells, colonies or in confluence. Other cell lines such as BEAS-2B or NE-18 also have characteristic cell-surface oligosaccharides (sialyl Le^a and Le^x respectively) that are expressed differently depending on the state of confluence. We considered the possibility that these varied expressions

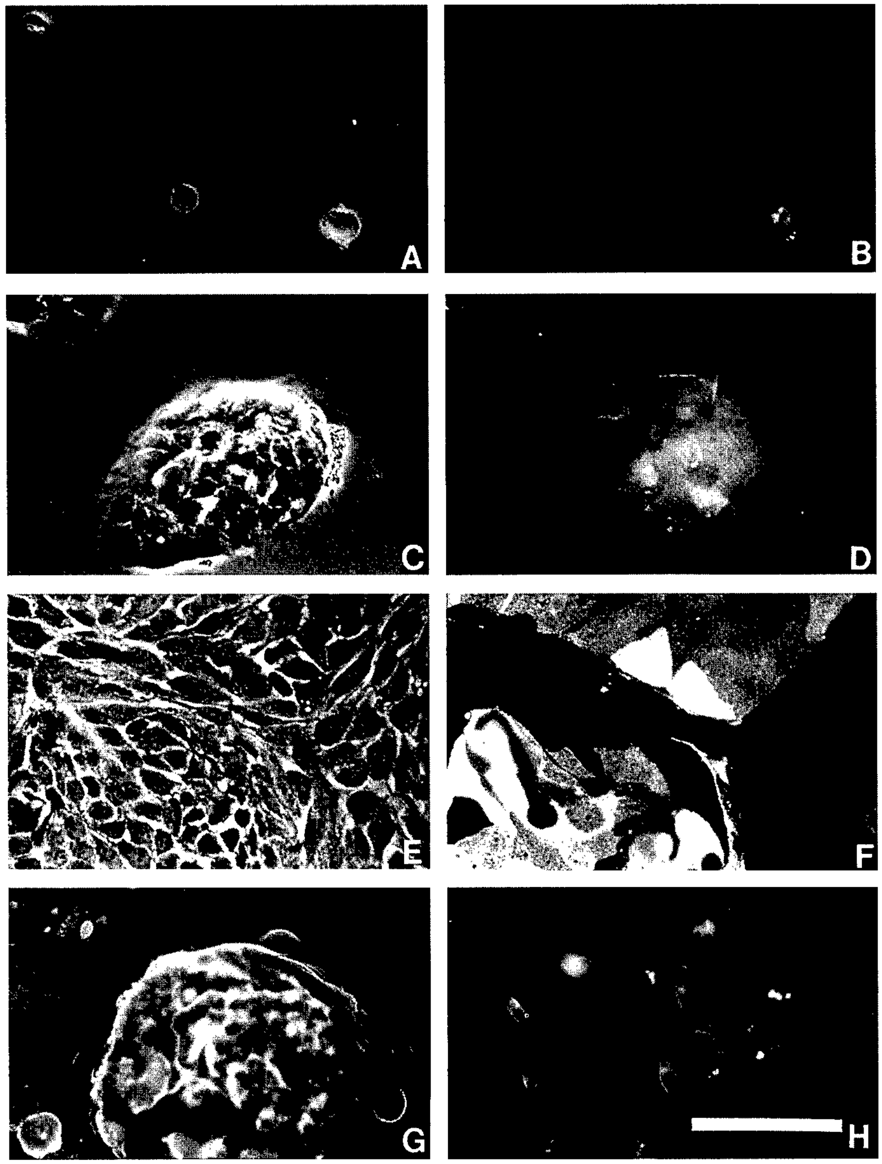

Figure 2. Variation in Le^a-Le^x expression by NU6-1 cells detected by Mab 43-9F. A and B. As single cells only 1 in 100 cells express Le^a-Le^x. A rare field is shown where two expressing cells occur. (A. Phase microscopy; and B. FITC immunofluorescent microscopy of the same cells.) C and D. In small colonies, expression of epitope is by cells at the periphery of each colony. (C. Phase microscopy; and D. FITC immunofluorescent microscopy of the same cells.) E and F. At confluence 30–50% of these cells express Le^a-Le^x and a geometric plate-like gel is apparent. (E. Phase microscopy; and F. FITC immunofluorescent microscopy of the same cells.) G. If these cells are allowed to continue to grow, with adequate new BCS supplemented media, 100% expressivity occurs. FITC immunofluorescent microscopy. H. 100% of single NU6-1 cells express the sialyl Le^a epitope. Of the panel of oligosaccharide epitopes studied sialylated Le^a is the only epitope expressed 100% of the time by single cells. FITC immunofluorescent microscopy. Bar, 150 μm, $\times$ 200.

could be influenced by the methods used to prepare single cell suspensions. For example, the first studies of NU6-1 used trypsin to release confluent cells, which were then plated down as single cells. However, we found that NU6-1 cells prepared by EDTA treatment, or single-cells spontaneously released as they entered mitosis, showed similar decreases in characteristic oligosaccharides, such as Le^a-Le^x when plated as isolated cells (Fig. 1A). Thus the changes in oligosaccharides seem to be characteristic of the cell-cell associations rather than the biochemical treatments that the cells receive.

At this point it is not clear if the observed changes in cell-surface oligosaccharides are attributable to altered regulation in glycosylation (for example via altered expression of glycosyltransferases) or to alterations in the cellular associations of glycoconjugates. An example of the latter would be a selective release into culture media of glycoconjugates carrying specific

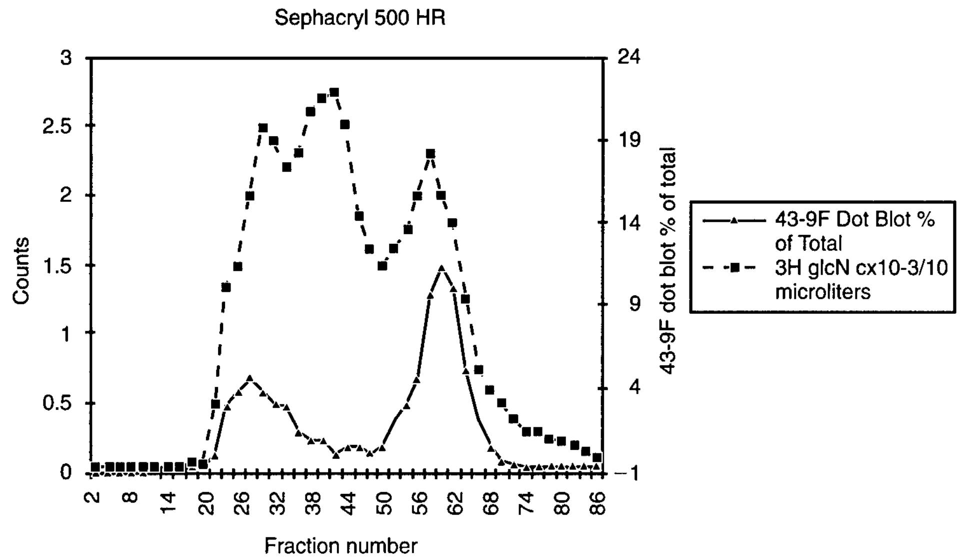

Figure 3. Le^a-Le^x expression by mucins and large glycoconjugates of cell line NU6-1. NU6-1 cells were allowed to grow to near confluence in FCS supplemented RPMI medium. The medium was removed and [^{3}H] glucosamine (10–3/10 μl) added to serum free medium. Growth continued for the next 72 h. The medium was harvested and large glycoconjugates precipitated with ammonium sulfate. These resultant glycoconjugates were applied to a Sephacryl S500 column and fractions collected. Immunoblots of each fraction was performed utilizing Mab 43-9F which identifies the Le^a-Le^x epitope. The oligosaccharide is present in association with the mucin peaks (fractions 18–42) as well as other large glycoconjugates (fractions 43–70).

oligosaccharides when cells grow without contacting neighbors. Alternatively, it also seems possible that mucins and other large glycoconjugates synthesized by one cell could be released and transferred to other neighboring cells and thus present the appearance of more extensive expression when cell densities are increased. These possibilities will be explored in future studies. However, preliminary studies have indicated that the transfer of mucins labeled with specific oligosaccharides does not readily occur from one cell type to another. It was demonstrated that soluble mucins and other large glycoproteins obtained from NU6-1 cells will not associate with the SLC-11 variant clone, NE-18, when the later is grown in media containing mucins decorated with labeled Le^a-Le^x and/or Le^a (data not shown).

Discussion

The present study confirms intracellular oligosaccharide diversity in cell lines (NU6-1 and NE-18) derived from the same tumor which behave very differently when studied in animal tumor models [20]. The results also extend those earlier findings to show that the oligosaccharide diversity on different cells is dependent on the density at which cells grow in tissue culture. Cell density dependent changes in a cell surface oligosaccharide were also observed in the BEAS-2B cell line, derived from normal lung cells, indicating that the phenomena applies to more than just tumor cell lines. It appears as though each cell type is capable of rapidly changing its cell-surface oligosaccharide perhaps either by regulating glycosyltransferase activities, or by modulating the associations of cell membrane glycocalyx quickly in response to associated cells.

It is not clear at this time if the changes in cell surface oligosaccharides that occur, as isolated cells divide and produce neighbors, are directly attributable to the cell-cell interactions that develop, or if the new cell interactions only initiate the changes indirectly through their effects on overall growth. However, it should be noted that the growth conditions, as reflected by cell division rates, do not change appreciably as small colonies develop from single, isolated cells. Yet, in some cases the cell-surface oligosaccharides change during this stage.

The results further suggest that the diversity of cell surface oligosaccharides seen in the developing tumors from these and other tumor cell lines may be at least partly attributable to differences in cell-cell contacts during tumor development. This could be an important consideration as tumor associated oligosaccharides are increasingly used for diagnostic or prognostic indicators.

Acknowledgements

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Decrease in Le^x Expression in Esophageal Adenocarcinomas Arising in Barrett's Epithelium[1]

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Abstract

Fifty esophageal adenocarcinomas were investigated for their expression of Le^a, Le^x, and Le^a-Le^x. Among the 50 adenocarcinomas, 17 cases developed in Barrett's epithelium. Those 17 differed from the other 33 cases by expressing much less Le^x. Fifty-nine percent of Barrett's adenocarcinomas were Le^x negative compared with 24% of the non-Barrett's carcinomas. All Barrett's adenocarcinomas showed less than 50% Le^x whereas 50% of non-Barrett's carcinomas showed between 50 and 100% expression. The statistical correlation coefficient for this association was $P < 0.001$. Normal gastric cardia epithelium showed the same Le^x expression in both groups. In the Barrett group, Le^x expression decreased from normal through intestinal metaplasia and dysplasia to adenocarcinoma. This progression was not seen in the non-Barrett group. Loss of Le^x expression may prove useful in following patients with Barrett's epithelium in evaluating progression toward a malignant process. No difference in expression of Le^a and Le^a-Le^x was found between Barrett's and non-Barrett's carcinomas.

Introduction

Carcinogenesis has been described as a molecular disease of cell membrane glycoconjugates (1, 2). Studies have demonstrated that although cell surface glycosylation varies with cell and tissue type, changes in relation to transformation have some common themes. These appear to be either incomplete oligosaccharide synthesis or neosynthesis (3–9).

Studies in one of our laboratories have focused on aberrant glycosylation of mucins and other large glycoproteins in non-small cell carcinomas of the lung and cancers of the gastrointestinal tract (10–12). Initially, we described expression of a previously unidentified extended Lewis antigen (Le^a-Le^x) in squamous cell lung carcinoma which is recognized by Mab[4] 43–9F. Le^a-Le^x is suspected to be exclusively associated with glycoproteins in contrast to other Lewis antigens which are glycoprotein/glycolipid carbohydrate moieties (13). These studies were extended to include the use of additional Mabs to identify expression of other Lewis antigens which have each, individually, been described as tumor-associated carbohydrate antigens by others (1, 14–17). Utilization of a panel of biomarkers on patient specimen was initiated in each case to increase the probability of concordant aberrant glycosylation which would lead to patterns which might have diagnostic and/or prognostic significance. Furthermore, emerging patterns could alter patient management decisions.

The current study focuses on adenocarcinomas developing at the gastroesophageal junction, either in Barrett's epithelium (which is columnar epithelium located in the distal esophagus generally believed to occur secondary to chronic reflux) or in non-Barrett's gastric cardia mucosa. Several investigations have shown that Barrett's epithelium is premalignant. These columnar epithelial cells transform first into dysplastic cells and then, in many instances, progress into adenocarcinomas (16–18).

We utilized Mabs against Le^a-Le^x, Le^a, and Le^x to assess expression in premalignant epithelium and within the adenocarcinomas arising in both gastric cardia mucosa and Barrett's epithelium.

Materials and Methods

Tumor Specimens. Slides from 50 esophageal adenocarcinomas (41 men and 9 women, ages 20–82 years) were obtained from the Department of Pathology, State University Hospital, Copenhagen Hospital Cooperation. The material consists of 17 adenocarcinomas developed in Barrett's epithelium and 33 other gastric cardia adenocarcinomas (1979–1990). All adenocarcinomas were surgical specimens. The diagnosis of esophageal adenocarcinomas was made if more than half of the tumor's length was above the gastroesophageal junction. The diagnoses of Barrett's esophagus in the 17 cases were based on the presence of Barrett's epithelium proximal to the gastroesophageal junction. From each surgical specimen, 15–30 blocks were taken representing resection lines, nontumorous mucosa, and pathological mucosa, whereas 1–10 blocks were taken of tumor and surrounding tissue. From each surgical specimen, one to six sections of nontumorous gastric cardia and one to three slides were chosen as representative. From the 17 Barrett cases, areas of Barrett's epithelium with intestinal metaplasia and, if present, dysplasia were chosen as well. Tumor differentiation in both groups included adenocarcinomas which were poorly, moderately, or well differentiated.

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[4] The abbreviation used is: Mab, monoclonal antibody.

Table 1 Le^x expression in 17 patients with Barrett's esophageal adenocarcinoma, dysplasia, intestinal metaplasia, and normal cardia and in 33 patients with non-Barrett's adenocarcinoma

Adenocarcinoma	% Positive cells	Barrett's adenocarcinoma (%)	Dysplastic (%)	Intestinal epithelium (%)	Normal metaplasia (%)	Non-Barrett's cardia (%)
	0	59	0	0	0	18
	1–10	18	71	59	18	18
	11–50	23	23	35	18	12
	51–80	0	6	6	64	30
	81–100	0	0	0	0	22

Antibodies. The three monoclonal antibodies recognizing Le^a (Co-514), Le^x [(P12)], and Le^a-Le^x (43–9F) were individually applied to each section, and both immunofluorescence and immunoperoxidase staining were performed on serial sections from each case. Each Mab was purified from serum-free culture media (RPMI 1640) of the respective hybridomas as described previously (6, 19). In some experiments, the antibody-containing media were used without purification. Purified Mabs were applied at a concentration of about 2 μg/ml (diluted in PBS), whereas Mabs in serum-free culture media were applied after dilution of 1:40 (in PBS).

For immunofluorescence, each tissue section was incubated with primary antibody at room temperature for 1 h in a humidity chamber. After 1 h the tissues were vigorously washed three times with PBS and secondary antibody was applied. For immunofluorescence, the secondary antibody was FITC- or tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse polyclonal IgG/IgM applied at a concentration of 1 μg/ml (Sigma, St. Louis, MO). Again, tissues were incubated for 1 h in humidity chambers at room temperature. After incubation, the tissues were thoroughly rinsed in PBS and several drops of antifade coverslip mounting media were applied to the sections, and the slides were coverslipped and stored in the dark at 4°C until they were evaluated.

For the immunoperoxidase technique, slides were preincubated at room temperature with PBS and BSA (15 min). Primary antibody was then applied and the sections were incubated overnight at 4°C. The peroxidase-conjugated secondary antibody, P260 (DAKO), was then applied in a dilution of 1:20, and the sections were incubated at room temperature for 60 min, developed in 0.04% 3-amino-9-ethylcarbazol for 10 min (Sigma), and counterstained with hematoxylin for 2 min. Aquamount coverslip media were applied and the sections were coverslipped.

On a routine basis, H&E sections were obtained adjacent to those sections which were evaluated using both immunohistology methods.

Evaluation. Evaluation of the patient specimen was on the basis of the fraction of positively stained tumor cells as well as the fraction of positively stained dysplastic or nondysplastic Barrett's epithelial cells and nontumorous gastric cardia mucosa. Semiquantitatively, each specimen was placed into one of the following groups: 0% positive cells, 1–10% positive cells, 11–50% positive cells, 51–80% positivity, and 89–100% positivity. The recording of positive tumor cells was based on all of the investigated tumor tissue, estimating the fraction and counting of 5–10 high-power fields. The intensity of the fluorescent reaction product for each was also evaluated each time (0–4+). Only reaction products of 4+ were recorded as positive. With each experiment, a known positive carcinoma control and a negative control were processed. Also, nonspecific reactivity was evaluated by paralleling a section with PBS, replacing primary antibody to each of the oligosaccharide epitopes.

Reevaluation of the tissues prepared for fluorescent microscopy was possible for up to 2 months, at which time the reaction product faded dramatically. Immunoperoxidase preparations are stable at room temperature for many years. These have been filed along with the patients original slides. Photomicrographs were taken on a Leitz Dialux 20 microscope with a UFX-II photographic attachment. Filter cube N for FITC incident light microscopy was used for immunofluorescent studies.

Statistical Analysis. Statistical evaluation of the data was performed using the Student t test. A two-tailed analysis was carried out. Only results with $P < 0.05$ were regarded as significant. These analyses were performed in the Biostatistics Core Laboratory of the University of Colorado Cancer Center.

Results

Le^x expression by tumors arising in Barrett's epithelium is markedly decreased when compared with adenocarcinomas arising in gastric cardia mucosa (Table 1). Fifty-nine percent of the Barrett-associated adenocarcinomas do not express this epitope, whereas only 18% of non-Barrett adenocarcinomas are negative. Additionally, when Le^x expression was present in the Barrett adenocarcinomas, the percentage of cells expressing the epitope was significantly reduced compared with the non-Barrett cases. All Barrett adenocarcinomas had less than 50% Le^x-positive tumor cells as shown in Fig. 1. Most of the non-Barrett cases contained between 50 and 100% of the tumor cells expressing Le^x, as demonstrated in Fig. 2. This difference between Barrett's and non-Barrett's cases was statistically significant ($P < 0.001$).

Gastric cardia epithelium in both patient groups expressed similar percentages of Le^x, and the areas of junctional type epithelium did not show any clear differences from nontumorous gastric cardia. Table 1 shows the progressive loss of the Le^x epitope by abnormal cells as intestinal metaplasia occurs and progresses to dysplasia in patients with Barrett's esophageal adenocarcinoma. The decrease of Le^x did not correlate with patient age or with tumor differentiation ($P > 0.5$). Moreover, we observed no morphological changes between Le^x-expressing cells and cells which were nonexpressors within the same tumor.

The present study also examined expression of Le^a and Le^a-Le^x by normal, metaplastic, dysplastic, and cancer cells of each of the 50 cases. Expression of Le^a and Le^a-Le^x was similar among both subsets of patients (results not shown).

Evaluation of patient specimens was undertaken by both fluorescence and immunoperoxidase staining to evaluate any differences each technique might reveal in terms of epitope expression. Immunofluorescence is usually considered more

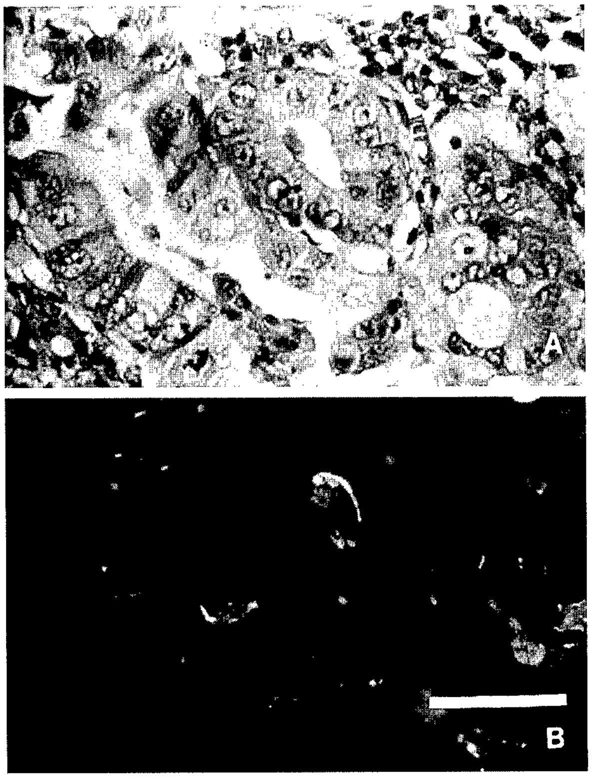

Fig. 1. Less than 50% of the adenocarcinoma cells arising in Barrett's esophagus are Le^x positive. *A*, H&E-stained section of a patient tumor and *B*, FITC immunofluorescent microscopy of adjacent serial section. ×200. *Bar*, 150 μm.

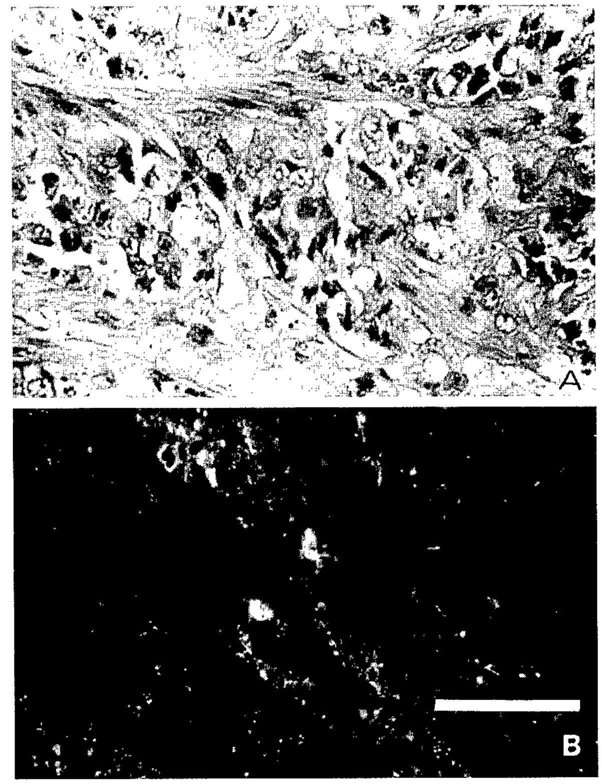

Fig. 2. Non-Barrett's adenocarcinomas (those not previously associated with reflux) contain tumor cells which are high in the expression of Le^x, 80% of these cells are Le^x positive. *A*, H&E-stained section of a patient tumor and *B*, FITC immunofluorescent microscopy of adjacent serial section. ×200. *Bar*, 150 μm.

sensitive compared with the immunoperoxidase technique which is more stable and may be more accurate when considering morphology. The fraction of positive cells was recorded identically with both techniques. Throughout the course of this study, parallel sections were independently evaluated by three different pathologists utilizing both techniques. Notably, no cases negative with the immunoperoxidase technique were scored positive with immunofluorescence and *vise versa*. Since the pathologists evaluating the different techniques evaluated only one technique or the other, there was no inter/intraobserver variation at this level.

Discussion

A number of anti-Lewis antibodies have been evaluated as probes for cancer markers (10, 20–22). Previously, changes in expression of Lewis antigens have been observed in human intestinal metaplasia, gastric adenomas and gastric carcinomas (9, 23, 24). Human gastric cancers show an enhanced expression of Le^a and loss of ABH (17). Distal colon cancers have been shown to express Le^b and Le^y aberrantly (7). Sialyldimeric Le^x, an oncodevelopmental carbohydrate antigen, has been shown to be expressed in human colorectal carcinomas, on both glycolipids and mucin proteins, and long and short chain Le^x antigens are significantly enhanced in colonic carcinoma (13). It has been postulated that several discrete cell populations at different stages of progression of tumors show variable patterns of glycosylation, and a single tumor can show mosaicism in the expression of carbohydrate antigens (14).

The present study corroborates the findings of others, expands the hypothesis of mosaicism, and confirms changes in epitope expression with epithelial transformation (23, 24). Furthermore, this study suggests possible differences in carbohydrate expression by malignant cells when tumors vary etiologically. Those adenocarcinomas of the distal esophagus which were preceded by intestinal metaplasia and dysplasia and clinically evolved secondary to chronic gastric reflux (Barrett's) contained subsets of transformed cells which progressively lost their Le^x cell surface epitope. Non-Barrett adenocarcinomas arising without documented Barrett's epithelium and symptoms which suggested the presence of gastric epithelium within the distal esophagus retained the expression of the Le^x molecule (Table 1). Documentation of the gradual decrease in Le^x expression from normal gastric cardia via intestinal metaplasia via dysplasia to invasive adenocarcinoma was possible in the Barrett cases. These changes are not present in the non-Barrett adenocarcinomas studied. In this analysis, it is possible that a Barrett-derived adenocarcinoma was misassigned if the carcinoma obliterated the preexisting metaplasia. Also, it is possible that a carcinoma arising in gastric cardia could invade nearby Barrett's epithelium and be incorrectly assigned. However, any misassignments should tend to blur any real differences in Barrett- and non-Barrett-derived adenocarcinomas. Such error in assignments, if they occur, would not be expected to artifactually create differences in marker expression that do not exist. Thus, the uncertainty should not compromise our general results.

Presently, it is unknown whether the decrease in Le^x expression occurs early in the evolution of Barrett's epithelium.

Longitudinal studies of patients with Barrett's esophagus are under way to explore further the evolution of these changes comparing junctional-type Barrett's epithelium with normal gastric cardia and specialized type to determine whether a decrease occurs in junctional epithelium and progresses through specialized epithelium. This study suggests that Le^x expression may be a useful tool in following patients with Barrett's epithelium, since a decrease of Le^x expression by these cells appears to herald the onset of progressive disease.

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